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ORIGINAL PAPER

Genome-wide transcription survey on flavour production in *Saccharomyces cerevisiae*

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Abstract The yeast *Saccharomyces cerevisiae* is widely used as aroma producer in the preparation of fermented foods and beverages. During food fermentations, secondary metabolites like 3-methyl-1-butanol, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoate and 3-methylbutyrate emerge. These four compounds have a major influence on the final taste of fermented foods. Their presence is influenced by the availability of free branched chained amino acids (BCAA). To study the underlying molecular mechanism of the formation of these compounds, we performed genome-wide transcription analyses with cDNA microarrays. The expression profile of yeast during flavour formation, when cultivated on L-leucine, was compared to the expression profile of cells cultivated on ammonia. In addition, the expression profiles of cells cultivated in a batch culture were compared to cells cultivated under continuous growth conditions. Genome-wide gene analysis of these samples revealed a group of 117 genes, which were more than two-fold up- or down-regulated and significantly altered in gene expression ($P < 0.001$) under both cultivation conditions. This group included genes encoding enzymes of different amino acid metabolism pathways. The group of the BCAA metabolism was not significantly altered in gene expression. Genes identified with altered expression levels, in only batch or continuous culture fermentions, represented functional groups concerning energy, protein fate, cell cycle and DNA processing. Furthermore, clustering of genome-wide data revealed that the type of cultivation overruled the differences in N-source in the gene-expression profiles. This observation emphasizes the importance of sample history in gene expression analysis.

Keywords cDNA microarray · Fermentation · Flavour · Fusel alcohols · Leucine metabolism · 3-Methyl-1-butanol · *Saccharomyces cerevisiae*

Introduction

Fungi are at the core of flavour formation during food fermentation. For example, the yeast *Saccharomyces cerevisiae* is responsible for the characteristic aroma profile, or so-called ''fermented taste'' in the production of bread and alcoholic beverages. The responsible products for this, as in all foods, consist of a complex mixture of flavour compounds. However, the characteristic impact of these fermented food flavours is determined by a small prominent group, consisting of fusel alcohols and their derivatives. Commonly known fusel alcohols are the substances: 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol (Meilgaard 1982; Reed et al. 1989).

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To predict, control and enhance the production of these ''character-impact'' compounds, profiling of yeast metabolic routes is of particular importance. When ammonia is used as nitrogen source, fusel alcohols can be synthesized via the isoleucine–valine–leucine (ILV) pathway (Derrick and Large 1993). However, their concentration drastically increases when BCAAs are present in the media. The dogma of fusel alcohol formation in yeast was already founded in 1907 (Ehrlich 1907). The ''Ehrlich pathway'' assumes the conversion of the branched chain amino acids to fusel alcohols by three enzymatic steps. The first step is a transaminase step, in which the amino group of the BCAA is transferred to 2-oxoglutarate, resulting in branched chain-oxoacids and glutamate. The second step is a decarboxylation step, which converts the branched chain oxoacids to branched chain aldehydes. And the last step is a reduction step, in which the branched chain aldehydes are reduced to branched chain alcohols or so-called fusel alcohols.

There are several questions concerning the Ehrlich pathway and fusel alcohol production. Firstly, the molecular mechanisms behind the production of flavour compounds remain unclear. Enzymes, involved in the synthesis of these flavours have been identified biochemically, but the identity and genes encoding enzymes involved in this pathway, are not fully elucidated. The only well-defined genes in the Ehrlich pathway are *BAT1* and *BAT2*, encoding branched chain amino acids transaminases, responsible for the first step (Dickinson and Norte 1993; Eden et al. 1996; Kispal et al. 1996). Both isozymes are able to catalyze the transfer reaction of the nitrogen group from leucine, or if available isoleucine and valine to 2-oxoglutarate. Although these genes were identified, they are not essential for fusel alcohol production by *S. cerevisiae* grown on glucose (Eden et al. 2001; Schoondermark-Stolk et al. 2005). Recent microarray data indicate that the function of Bat1p and Bat2p differ with the available carbon sources (Schoondermark-Stolk et al. 2005). Concerning the other Ehrlich pathway steps, it has been proposed that in particular PDC-like enzymes in *S. cerevisiae*, coded by *YDL080c* and *ARO10*, are involved by the decarboxylation of 2-oxopentanoate (Dickinson et al. 2003; Vuralhan et al. 2003, 2005). An NADP-dependent branched chain Adh (bcADH) might be responsible for the reduction of the branched chain aldehyde to the fusel alcohol (van Iersel et al. 1997). Two genes have been identified, *YMR318c* (*ADH6*) and *YCR105w* (*ADH7*), encoding NADP(H)-dependent alcohol dehydrogenases (Larroy et al. 2002, 2003). However, clear evidence about the identity of the responsible *ADH* is not available.

The second inadequacy of the Ehrlich pathway is the generation of different oxo-acids and fusel alcohols from branched chain amino acids, which cannot be explained by a linear pathway. Prior to this research, no genes were identified to increase fusel alcohol production (De Boer et al. 1998; ter Schure et al. 1998). With the development of cDNA microarrays, a tool has become available to identify or better understand the expression of genes that function in metabolic pathways or as regulatory genes for that pathway (DeRisi et al. 1997; Marshall and Hodgson 1998). This study was performed to generate new clues on the identification of genes involved in fusel alcohol production.

Materials and methods

Yeast strains and maintenance

The isogenic wild-type *S. cerevisiae* strain CEN-PK 113-7D was used for fermentation experiments and gene expression analysis. Frozen stock cultures containing 20% w/v glycerol were stored at −80°C. Working stocks were maintained on YP-agar slants containing 20 g/l glucose.

Chemostat cultivation for batch growth

Inoculum cultures were grown for approximately 48 h in Erlenmeyer flasks at 30°C in a rotary shaker (180 rev/min) on 1.7 mg/ml yeast nitrogen base without amino acids and without ammonium sulphate (YNB; DIFCO Laboratories, Detroit, USA), supplemented with 100 mM glucose and 15 mM L-leucine to an OD_{600} of 1.0 (±0.2). The cell suspension (100 ml) was transferred to 1.0-l fermentors (Infors, Bottmingen, Switzerland), with a working volume of 500 ml. Two independent chemostat batch-fermentations were run in order to obtain independent duplicates. Air was supplied in the chemostats at a flow rate of 20 l/h, while temperature was set at 30°C. The dissolved oxygen concentration was always above 25%. pH was maintained at 5.0 by automatic titration with 1 M KOH, containing 1% (v/v) Struktol® as anti-foaming reagent. Stirrer speed was set at 500 rev/min. Biomass was generated during controlled batch cultivation on glucose containing minimal medium (100 mM glucose, 1.67 g YNB l^{-1} without amino acids and ammonia). The branched chain amino acid, L-leucine, was used as

nitrogen source with a final concentration of 15 mM. Control cells were cultivated on ammonium sulphate as nitrogen (N)-source, with a final concentration of 15 mM NH_3. Off-gas analysis was performed using a PRIMA 600 gas analysis mass spectrometer (VG-gas, Winsford, Cheshire, UK). During cultivation, samples for off-line analyses were taken from the bioreactor: 10 ml of a fresh sample was taken and centrifuged for 5 min at 4,000 rev/min, 5°C. The supernatant was used for analysis of extracellular substrates and metabolites. The remaining pellet was used for determining the biomass dry weight. Fresh sample was also used for determination of the optical density and preparing samples for fusel alcohol measurements. For the latter 100 μl sample was taken and 300 μl methyl formate was added.

Chemostat cultivation for CSTR

Inoculum cultures were grown for approximately 48 h in Erlenmeyer flasks at 30°C in a rotary shaker (180 rev/min) on 1.7 mg/ml yeast nitrogen base without amino acids and without ammonium sulphate (YNB; DIFCO Laboratories, Detroit, USA), supplemented with 100 mM glucose and 15 mM L-leucine to an OD_{600} of 1.0 (±0.2). The cell suspension (100 ml) was transferred to a 2-l BiofloIII fermentor (New Brunswick Scientific, Nijmegen, the Netherlands), connected to a computer controller unit running with Advanced Fermentation Software (New Brunswick Scientific, Nijmegen, the Netherlands). After overnight growth carbon-limited chemostat cultivation was performed at a temperature 30°C and a stirrer speed of 800 rev/min. The culture pH was maintained at 5.0 by automatically controlled addition of 1 M KOH. The yeast was grown at a dilution rate of 0.10 per hour with a feed containing 15 mM L-leucine and 20 g glucose per liter on EGLI culture medium (Meijer et al. 1998). Continuous feed was connected after overnight batch growth. The working volume was kept at 2 l by continuous removal of effluent and the airflow was also kept constant. Carbon dioxide production and oxygen consumption were monitored online by a Servomex 1440 Gas Analyser (Servomex B.V., Zoetermeer, the Netherlands). The ethanol production was monitored by a Servomex gasanalyser 2500 Single Beam Infrared. During cultivation samples for off-line analyses were taken from the bioreactor: 10 ml of a fresh sample was taken and centrifuged for 5 min at 4,000 rev/min, 5°C. The supernatant was used for analysis of extracellular substrates and metabolites. The remaining pellet was used for determining the biomass dry weight. Fresh sample was also used for determination of the optical density and preparing samples for fusel alcohol measurements. For the latter 100 μl sample was taken and 300 μl methyl formate was added. Two independent chemostat continuous-culture fermentations were run in order to obtain independent duplicates.

Determination of culture dry weight and cell amounts

For the biomass dry weight determination, the pellet, obtained as described above, was washed with demineralized water and centrifuged again. Next, the pellet was dried overnight at 80°C and weighted. The attenuance (OD_{600}) of the medium was determined on a spectrophotometer (Ultrospec 3000, Amersham Biosciences, Roosendaal, the Netherlands).

Substrate and metabolite analysis

Enzymatic determination of the initial and residual glucose concentration was performed using a Cobas Mira S autoanalyser (Roche Diagnostics Nederland B.V., Almere, the Netherlands) measuring $NADPH_2$ formation with an enzyme mix of hexokinase and glucose-6-phosphate dehydrogenase (Glucose HK 125, ABX diagnostics, Montpellier, France). 3-Methyl-1-butanol and L-leucine concentrations were determined by GC and HPLC, respectively, as described previously (Schoondermark-Stolk et al. 2006). All measurements were performed in duplicate.

Cell extract preparation and synthesis of cDNA

Cells were harvested for cDNA microarray analysis in the late exponential phase during batch growth, and in the continuous culture grown cells, after a steady state had been reached and the bioreactor had been refreshed with at least two times its own volume. Samples were taken from the two independent duplicate fermentation cultures. Cells were harvested from batch cultures at $t = 24$ h or from continuous culture steady state samples and total RNA was isolated as described previously (Schoondermark-Stolk et al. 2002). A small amount of RNA from each sample was loaded on a microgel, electrophoresed, scanned and analysed for the quantity and integrity of the 18S and 28S ribosomal RNA bands, to ensure the quality of the isolated RNA (Bioanalyzer 2100, Agilent Technologies, Palo Alto, CA). Next, synthesis of [^{33}P]CTP-labelled cDNA and GeneFilter® (Invitrogen ResGen, Breda, The Netherlands) hybridization were performed as described previously (Schoondermark-Stolk et al. 2002).

Data analysis and spot validation

Images were scanned at 50 micron resolution in ImageQuant and were imported into the ImaGene® microarray analysis software (BioDiscovery Inc., El Segundo, CA, USA). ImaGene® was used for a preliminary quantification of spot intensities to compare gene filter images pair wise. To determine the extent of induced or repressed gene-expression, all spot intensities were normalized against the total spot intensity. Each hybridization spot appearing was averaged and validated (Schoondermark-Stolk et al. 2002). The web-accessible gene expression analysis tool SNOMAD (Standardization and NOrmalization of MicroArray Data) was used to correct for bias and variance which are nonuniformly distributed across the range of microarray element signal intensities (Colantuoni et al. 2002). Two non-linear transformation calculations were performed for the local mean normalization and for local variance correction (Z-score generation using a locally calculated standard deviation), in order to correct hybridization artifacts resulting from the radioactive hybridization + washing processes (manifested as non-uniform background intensities). Microarrays were performed in quadruplicate (two microarrays for each duplicate sample). To identify significantly altered gene expression and reduce the number of false positives the program Significant Analysis of Microarrays (SAM) was used (Tusher et al. 2001). This program compared the difference in gene expression within the quadruplicates to that of the other samples. Ammonium batch cultured cells were compared with L-leucine-batch-cultured cells and the ammonium continuously cultured cells were compared with L-leucine continuously cultured cells.

Results and discussion

Growth and flavour formation in batch and continuous cultures

Batch growth was followed over 8 days (Fig. 1). There was no significant difference in lag-phase between batch cultures grown on L-leucine or ammonium sulphate as sole N-source.

Four volatile flavour substances; 3-methyl-1-butanol (isoamyl alcohol), 4-methyl-2-oxopentanoate (α-keto isocaproate), 3-methyl-2-oxobutanoate (α-keto isovalerate) and 3-methylbutyrate (isovaleric acid), were detected in the supernatant of L-leucine-cultivated cells (Fig. 2).

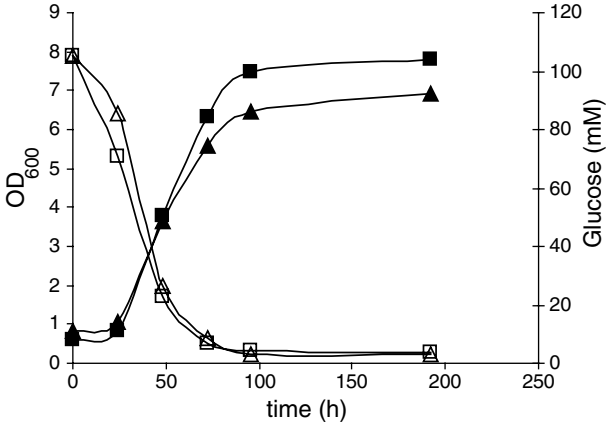

Fig. 1 Growth curve and glucose consumption of *S. cerevisiae* cells, batch-cultivated in 0.5 l on minimal medium with 7g/l glucose. OD_{600} of cells cultivated on ammonium sulphate (■), OD_{600} of cells cultivated on L-leucine (▲), Glucose concentration of cells cultivated on ammonium sulphate (□), Glucose concentration of cells cultivated on L-leucine (Δ)

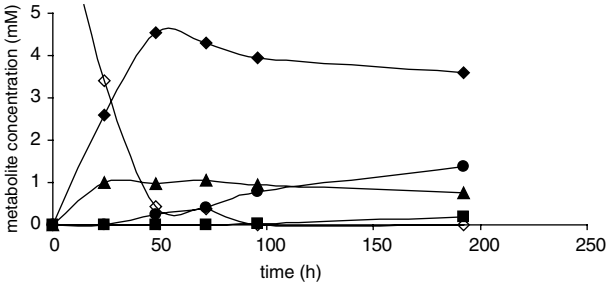

Fig. 2 Production of volatile metabolites and L-leucine consumption of *S. cerevisiae* cells, batch-cultivated in 0.5 l on minimal medium with 7 g glucose/l and 15 mM L-leucine (◇). 3-Methyl-1-butanol (◆), 4-methyl-2-oxopentanoate (▲), 3-methyl-2-oxobutanoate (■) and 3-methylbutyrate (●)

Cells started to produce 3-methyl-1-butanol and 4-methyl-2-oxopentanoate immediately after inoculation. After 24 h the metabolite 3-methylbutyric acid was detected in the medium. After 48 h the highest 3-methyl-1-butanol concentration of 4.54 mM was measured. After 192 h the concentration had declined to 3.59 mM, most probably caused by evaporation. The other metabolites, important for flavour determination due to their penetrating aroma, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoic acid, and 3-methylbutyric acid reached concentrations of 1.05, 0.14 and 1.38 mM, respectively. None of these products were detected in the supernatant of ammonium sulphate-cultivated cells. 4-Methyl-2-oxopentanoate is regarded as the first product in the L-leucine degradation pathway. The detected 3-methyl-1-butanol is known as the final product of the L-leucine degradation pathway. The other detected products, 3-methyl-2-oxobutanoic

acid and 3-methylbutyric acid, are not known to be involved in this pathway.

In CSTR-cultured cells, the same flavour substances, 3-methyl-1-butanol, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid emerged. A concentration was measured in the medium of 0.4 mM 3-methyl-1-butanol, 0.25 mM 4-methyl-2-oxopentanoate, 0.1 mM 3-methyl-2-oxobutanoic acid and 0.025 mM 3-methylbutyric acid. Also under these conditions, no flavour substances were detected in the medium with ammonium sulphate as N-source.

Genome-wide survey on gene expression of L-leucine- versus ammonium-grown cells

Cells were harvested for microarray analysis in the late exponential phase (batch cultures) or from steady state conditions (continuous growth cultures). Spot intensities of the microarrays were first normalized by SNOMAD (Colantuoni et al. 2002) for background correction and correction of error prone filter hybridization or spot imaging. After normalization, low expression values were removed and ^{2}log transformations were applied. ^{2}Log ratios of gene expression levels were analysed with the SAM-test (Tusher et al. 2001) to identify genes significantly altered in gene expression ($P < 0.01$ and $-1 > {}^2\log(\text{ratio}) > 1$). In batch cultured cells 930 genes were identified with a significantly altered gene expression when cultivated on L-leucine, compared to that of cells cultivated on ammonia. In the other experiment, cells cultivated in a CSTR under controlled pH, aeration and dilution rate, 752 genes showed significantly altered gene expression. Of the 930 genes, 813 genes showed altered expression specifically in the batch-cultured cells and 117 genes showed similar altered expression in batch as well as in continuous cultured cells. Concerning the CSTR cells, 635 genes of the 752 genes showed altered expression, which were not found in the gene group of batch-cultured cells. The three gene groups, 635 genes of the continuous culture, 117 genes of both cultures and 813 genes of the batch culture were further categorized, according to the MIPS database (Mewes et al. 2000). These analyses revealed that most genes with affected expression levels have functions in cell cycle and DNA processing, energy, metabolism, protein synthesis or fate, rescue and defence, transcription, transport or are classified as proteins with unknown biological functions (Table 1).

Further partitioning of genes associated with metabolism revealed that the majority of the overlapping metabolism genes, 82%, were specifically involved in amino acid metabolism. To establish the significance of the presence of identified gene groups, SGD GO TermFinder was used (Ashburner et al. 2000) for further analysis of the data. 69 gene ontology (GO) groups were identified with GO TermFinder as significantly represented function groups within the group of 117 genes ($P < 0.01$). These functional groups included genes involved in the amino acid biosynthesis, amine biosynthesis, caboxylic acid- and organic acid metabolism (*ARG1, BAT1, ACH1, GCN4, LYS1, CIT1, CIT2, DLD3, ARO3, GCV1, HOM3, ARG3, ARO4, SER33, HOM2, PDA1, ARO8, LYS21, ARO2, GLN1, ARG4* and *TRP4*). Separately mentioned amino acid metabolism-groups were the group of aromatic amino acids (*ARO3, ARO4, ARO8, ARO2* and *TRP4*), serine, homoserine and threonine (*GCV1, HOM3, SER33* and *HOM2*), arginine and glutamine (*ARG1, GLN3, GDH2, ARG3, GLN1* and *ARG4*).

The similar altered gene expression profiles in yeast under the two different growth conditions may indicate similar functions of the genes during batch or continuous growth. Under both conditions, four volatile flavour substances were produced, correlating the appearance of he aroma compounds with the altered gene expression. Major affected gene expression groups consisted of genes encoding proteins involved in amino acid metabolism. This indicates that amino acid metabolism pathways, other than the BCAA pathway, play significant roles in the formation of volatile flavours, independently of growth rate or other variation in cellular environment.

Table 1 Gene groups with common biological functions according to MIPS database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html) among the transcripts that were altered in expression, comparing L-leucine-grown cells with ammonia-grown cells

Function	Batch	CSTR	Overlapping
Cell cycle and DNA processing	73 (9%)	35 (6%)	2 (2%)
Energy	54 (7%)	48 (8%)	15 (13%)
Metabolism	137 (16%)	120 (18%)	28 (23%)
Miscellaneous	37 (5%)	77 (12%)	3 (3%)
Protein synthesis or fate	183 (22%)	81 (13%)	15 (13%)
Rescue and defence	38 (5%)	43 (7%)	11 (9%)
Transcription	110 (13%)	49 (8%)	10 (9%)
Transport	79 (10%)	77 (12%)	9 (8%)
Unclassified Proteins	102 (13%)	105 (16%)	24 (23%)
Total	813 (100%)	635 (100%)	117 (100%)

Miscellaneous: cellular communication, signal transduction, regulation of interaction with cellular environment, cell fate or viral and plasmid proteins

Identification of regulatory elements

Genes with similar expression patterns often have common regulatory elements in their promoter region. These elements are bound by similar transcription factors. Concerning N-metabolism, several upstream activation sequence (UAS) elements have been discovered. UAS_{NTR} for example, consists of two separate dodecanucleotide sites with the sequence GAT(T/A)A at their core (Bysani et al. 1991) and genes containing this element can be induced or repressed via this element. To detect overrepresented elements in the promoter regions of the 117 selected genes, the program Regulatory Sequence Analysis Tools (RSAT) was used. RSAT provided a series of modular computer programs specifically designed for the detection of known regulatory signals in non-coding sequences (van Helden 2003). Two regulatory elements were significantly present within the group of 117 genes; the Gcn4 element (GAGTCA; $p = 3.3^{-5}$) and the Gln3 element (GAT(T/A)A; $p = 2.5^{-5}$). The transcription factor Gcn4 was descibed as regulator in the general response to amino acid starvation (Natarajan et al. 2001). *GCN4* was upregulated by growth on L-leucine compared to ammonia and showed a more than 4-fold upregulation in the batch and continuous culture. *GLN3*, encodes a GATA-type zinc finger transcription factor for positive N-regulation (Cox et al. 2000). The *GLN3* gene is required for the activation of transcription of a number of genes in response to the replacement of ammonia by leucine as source of nitrogen, including the general amino acid permease *GAP1* (Magasanik 1992). The microarays showed high hybridization spots for both genes *GLN3* and *GAP1* of cDNA from cells cultivated on L-leucine. The expression levels of other genes encoding transcription factors involved in nitrogen metabolism, *LEU3, SKP1* and *MET30*, were significantly reduced on L-leucine growth. There was no significant discrepancy in expression ratios in this group due to the difference in cell cultivation, batch or CSTR.

Cluster analysis of genome-wide and L-leucine specific expression patterns

We applied hierarchical clustering on the four datasets; a genome-wide clustering (Fig. 3A) and clustering on the data aggregation from the SAM-test (group of 117 genes, Fig. 3B), to depict biological order in the gene expression response.

As is seen in Fig. 3A and B, in general the influence of cultivation methods on gene expression can be distinguished by a ''cultivation-dendrogram''. The dendrogram explicitly reveals the impact of the difference in cultivation method, batch or CSTR, above the difference in N-source. Thus genome-wide, the formation of flavour compounds makes little difference in the observed pattern of gene expression in comparison to CSTR or batch cultivation. This result shows that gene profiles, compared across different fermentation conditions, do not contribute significantly to the identification of logical correlations. On the other hand, clustering of the group of 117 genes shows a different dendrogram, resulting in the clustering of the two L-leucine cultivated cell experiments (Fig. 3B). Therefore, clustering of the relatively small number of genes, obtained via non-identical conditions, demonstrates the value of the SAM-identified relevant genes and strengthens the indication that these genes are particularly involved in flavour formation.

Transcriptional response of genes, specifically involved in flavour formation

Previously, the family of permeases was described which are responsible for the uptake of L-leucine (Forsberg et al. 2001). Yeast cells cultivated in the presence of L-leucine, genes encoding amino acid permeases or ammonium permease were affected during flavour-forming conditions. On the arrays, probes of *GAP1, BAP2, BAP3, ALP1, AGP2, TAT1* and *DIP5* showed strong hybridization signals with either batch or CSTR-cultured samples, but only during flavour formation (Fig. 4).

The genes *BAP2, BAP3* and *GAP1* contain all three an UAS for Gln3. Other enzymes, two branched chain transaminases (*BAT1* and *BAT*) are identified as enzymes responsible for the transaminase step in the Ehrlich pathway. Induced expression of *BAT1* was found in both culture conditions grown on L-leucine, whereas expression of *BAT2* was only observed in the L-leucine continuous culture cells. An explanation for undetected transcripts of *BAT2* in batch cultured cells may be that *BAT2* expression is growth-rate-dependent and induced during stationary growth phase, similar as *HXT5* (Eden et al. 1996; Verwaal et al. 2002). In addition, it has been described that *BAT2* has a minor role in cells, when cultivated on glucose whereas the presence of *BAT2* is essential during growth on ethanol (Schoondermark-Stolk et al. 2005). Concerning the second step of the Ehrlich pathway, there are indications that pyruvate decarboxylases might be responsible for the decarboxylase step of the Ehrlich pathway. Expression patterns of genes encoding pyruvate decarboxylases and pyruvate decarboxylase-like proteins: *LPD1, PDA1, PDB1, PDC1,*

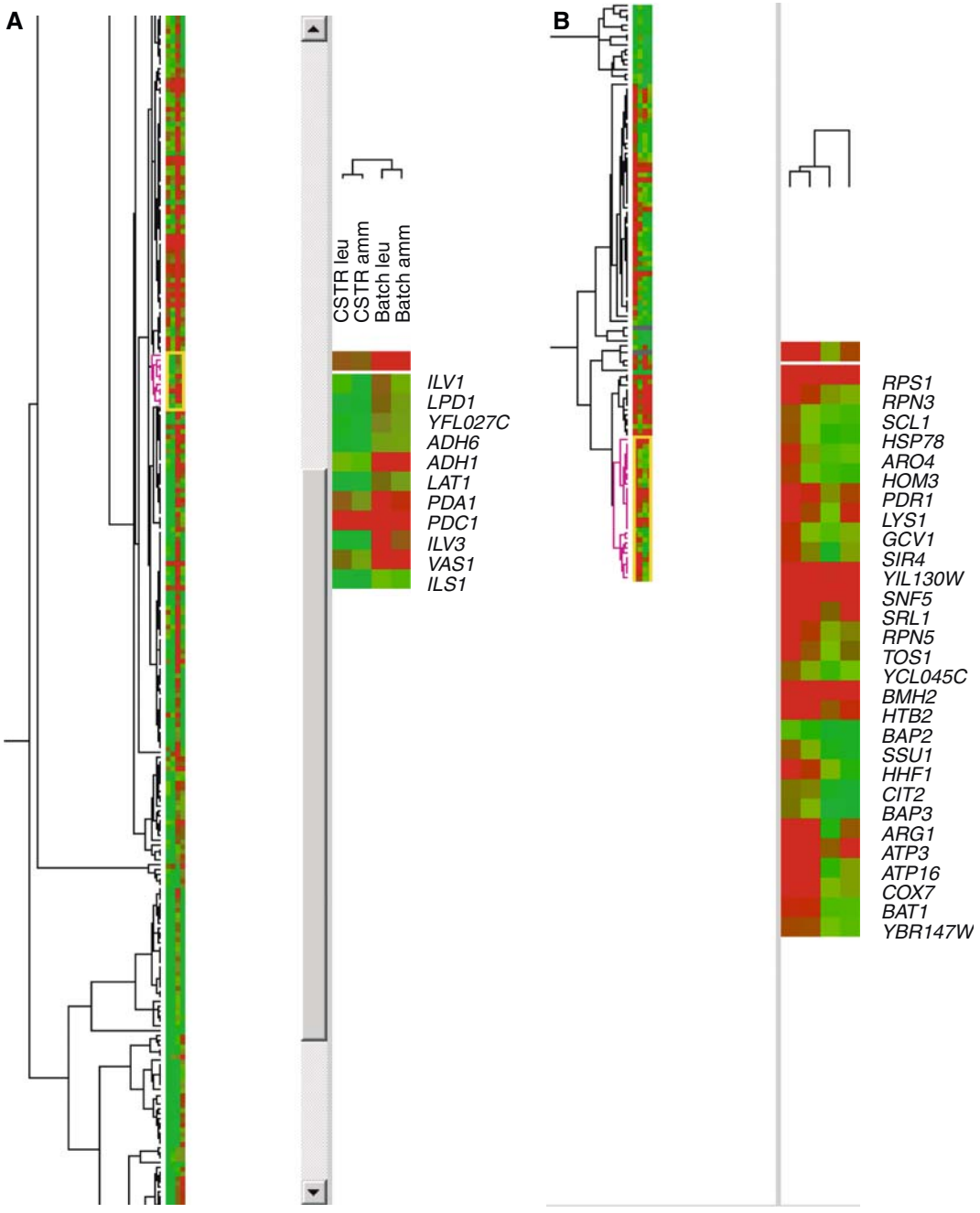

Fig. 3 (**A**) Hierarchical clusters of the four datasets visualized by Treeview. Genome-wide clustering shows a cluster dendrogram for the datasets. The two datasets from samples from CSTR- and the two datasets from batch cultivation are clustered together, visualized by the horizontal dendrogram. (**B**) Hierarchical clusters of the four datasets visualized by Treeview. Clustering on the specific group of 117 genes shows a dendrogram, clustering cells, cultivated on L-leucine together. CSTR-cultivated cells on ammonium sulphate displayed an expression pattern, apparently different to that of the batch cultivated cells

PDC5, PDC6 and *YDL080c* did not show a significant difference in gene expression during 3-methyl-1-butanol production, except for *ARO10*. *ARO10*, identified as a phenylpyruvate decarboxylase gene with a broad 2-oxo acid decarboxylase activity (Verwaal et al. 2002; Vuralhan et al. 2003, 2005) was strongly induced during flavour formation. In addition *PDX1*, pyruvate dehydrogenase complex protein X, the binding protein for Lpd1p, showed a significant increase in gene expression at the same conditions. This gene product plays an important role in the decarboxylation of branched chain oxoacids (Dickinson et al. 1997) and

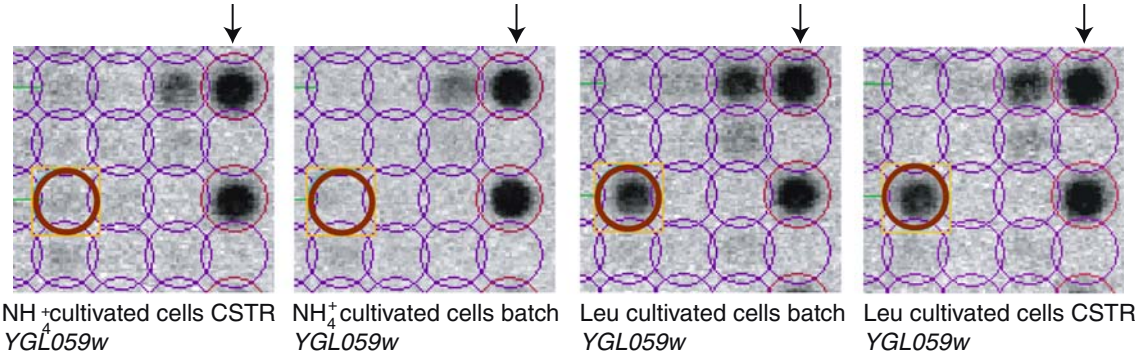

Fig. 4 *YGL059w* expression in batch or CSTR culturing. Arrows indicate control hybridization spots, included as reference (100%). *YGL059w* gene-expression was observed in both batch and CSTR when L-leucine was present in the medium as N-source

may catalyze the decarboxylation of 4-methyl-2-oxopentanoate to 3-methylbutyraldehyde. The last step of the Ehrlich pathway concerned a reduction step by via an alcohol dehydrogenase producing isoamyl alcohol out of the aldehyde (Derrick and Large 1993). Also none of the alcohol dehydrogenases showed a significantly altered gene activity. Transcripts of *YMR318c*, proposed to be involved in the synthesis of fusel alcohols (Larroy et al. 2002), were present and could be clearly detected in the cell. However, the gene showed no significant change in its activity during the flavour fermentation. Another gene, *YCR105w*, also a NADPH-dependent alcohol dehydrogenase, with a 64% protein similarity to *YMR318c* was highly induced during leucine CSTR cultivation.

Interestingly, *S. cerevisiae* possesses two ORFs with similarity to human branched chain oxoacid dehydrogenase kinase, *YIL042c* and *YGL059w*. *YGL059w* showed a strong hybridization signal. The presence of a functional BC 2-oxoacid dehydrogenase complex in yeast can clarify the presence of metabolites like 3-methylbutyric acid via the formation of isovaleryl CoA. In its turn isovaleryl CoA could be further converted into isovaleric acid by an acetyl CoA hydrolase. The acetyl CoA hydrolase gene, *ACH1*, was significantly upregulated in CSTR cultivated cells, when grown on leucine. If the gene expression reflects proteins involved in this pathway, the presence of 3-methylbutyrate (isovaleric acid) can be explained by the existence of a functional BC oxoacid dehydrogenase complex (Fig. 5).

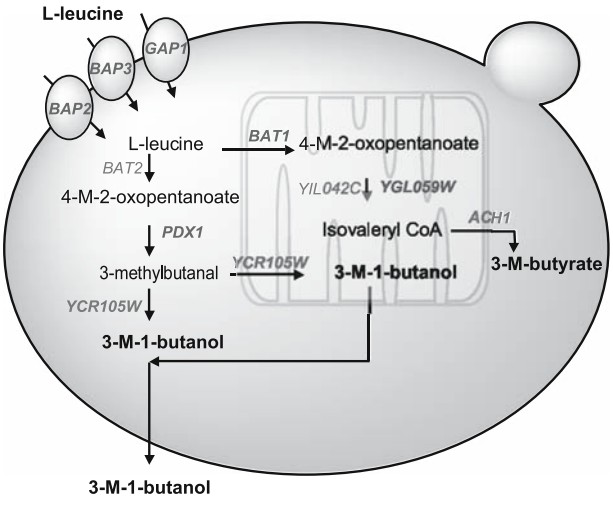

Fig. 5 Schematic representation of metabolic routes of L-leucine, leading to 4-methyl-2-oxopentanoate, 3-methyl-1-butanol and 3-methylbutyrate. Genes, enhanced in expression in one of the investigated condition are depicted. Genes, enhanced in expression in both, batch and CSTR culturing conditions, are depicted in bold

Conclusions

Flavour formation during food fermentation has been studied for decades, since it was discovered that microorganisms were responsible for the generation of aroma substances. High impact aroma molecules were derived from the conversion of different amino acids. Most enzymes involved in the metabolic pathway of these flavours have been identified biochemically (Dickinson and Norte 1993). However, the biological mechanisms behind the production of flavour compounds have not yet been elucidated. The conversion of branched chain amino acids into their respective oxoacids and alcohols is mostly studied via *BAT1* and *BAT2* deletion and over-expression studies. These studies showed that the branched chain transaminases Bat1p and Bat2p catalyse the deamination of L-leucine into the oxoacids, but they are not essential for the production of the high impact flavour 3-methyl-1-butanol (Eden et al. 2001; Schoondermark-Stolk et al. 2005). The following steps of the Ehrlich pathway are less extensively studied and prior to this research, no genes were identified that increased the production of 3-methyl-1-butanol.

Due to the development of cDNA microarrays, a tool is provided study gene-expression genome-wide. The expression analyses show expression of genes without preceding selections.

In this study we have shown that cell cultivation methods, regardless of flavour production connected to N-metabolism, have a major influence on the transcriptome. However, a group of genes with an expression profile correlated with the formation of aroma compounds have been identified by microarray expression analyses. Clustering of the expression profiles of these 117 genes surpassed the influence of the cultivation methods and resulted in 2 clusters of L-leucine- or ammonia-cultivated cells. Therefore, these genes are identified as specifically affecting flavour formation arising from L-leucine. The group consists of genes encoding enzymes involved in the amino acid metabolism of aromatic amino acids, serine, arginine, glutamine, homoserine and lysine, amine biosynthesis, caboxylic acid and organic acid metabolism. Previous studies of the soy yeast *Zygosaccharomyces rouxii* showed the interference of aspartate-derived amino acid metabolism when threonine was added to the medium (van der Sluis et al. 2000). Also our results show connections of apparently unconnected pathways: carboxylic acid or other amino acid metabolisms.

Furthermore, genes with regulatory elements for Gcn4 and Gln3 were significantly present in the group of affected genes. Gcn4p, a basic leucine zipper protein, is identified as primary regulator of the transcriptional response to amino acid starvation (Natarajan et al. 2001). Gcn4p is required for the full induction of at least 539 genes, involved in every amino acid biosynthetic pathway except cysteine, and genes encoding amino acid precursors, vitamin biosynthetic enzymes, peroxisomal components, mitochondrial carrier proteins, and autophagy proteins (Natarajan et al. 2001). Identification of Gcn4p, involved in flavour formation, which is closely related to the amino acid pathways is in agreement to previous transcription profiling results. *GLN3* gene of *S. cerevisiae* is required for the transcription of a number of genes in response to the replacement of glutamine by glutamate. Immunoprecipitation experiments indicated that the Gln3-protein binds the nitrogen UAS of *GLN1*, the gene encoding glutamine synthetase (Minehart and Magasanik 1992). Either the control of transcription or control of initiation of translation of *GLN3* and *GCN4* genes and target genes are therefore important for the formation of volatile flavours in response to L-leucine conversion.

In addition, *BAP2* and *BAP3*, which are required for the uptake of L-leucine, *ARO10*, *PDX1* and *YDL080c*, encoding decarboxylases for 4-methyl-2-oxopentanoate, *YMR318c* and *YCR105w*, encoding an alcohol dehydrogenase, genes homologous to leucine degradation genes in mammals and bacteria, were upregulated in *S. cerevisiae* during flavour formation. *YGL059w* an ORF with similarity to human branched chain oxoacid dehydrogenase kinase, was also upregulated. *YGL059w* has been suggested to be involved in branched chain amino acid degradation via the formation of the volatile flavours, 3-methyl-1-butanol, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid, through an oxidative decarboxylation route (Fig. 5).

Therefore, microarrays have a valuable contribution in the elucidation of the metabolism behind flavour formation. They provide new insights and reveal previously unnoticed connections. Further identification of the enzymes and genes involved, may ultimately lead to the optimization and control of a desired blend of flavours in fermented foods.

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